Modulation of voluntary ethanol consumption by beta-arrestin 2

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ABSTRACT **Beta-arrestin 2 is a multifunctional key component of the G protein-coupled receptor complex and is involved in μ-opiate and dopamine D2 receptor signaling, both of which are thought to mediate the rewarding effects of ethanol consumption. We identified elevated expression of the beta-arrestin 2 gene (*Arrb2*) in the striatum and the hippocampus of ethanol-preferring AA rats compared to their nonpreferring counterpart ANA line. Differential mRNA expression was accompanied by different levels of Arrb2 protein. The elevated expression was associated with a 7-marker haplotype in complete linkage disequilibrium, which segregated fully between the lines, and was unique to the preferring line. Furthermore, a single, distinct, and highly significant quantitative trait locus for *Arrb2* expression in hippocampus and striatum was identified at the locus of this gene, providing evidence that genetic variation may affect a *cis*-regulatory mechanism for expression and regional control of *Arrb2*. These findings were functionally validated using mice lacking *Arrb2*, which displayed both reduced voluntary ethanol consumption and ethanol-induced psychomotor stimulation. Our results demonstrate that beta-arrestin 2 modulates acute responses to ethanol and is an important mediator of ethanol reward.—Björk, K., Rimondini, R., Hansson, A. C., Terasmaa, A., Hyytiä, P., Heilig, M., Sommer, W. H. Modulation of voluntary ethanol consumption by beta-arrestin 2. *FASEB J.* 22, 2552–2560 (2008)**

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ARRESTINS ARE CYTOSOLIC PROTEINS that bind to guanine nucleotide binding protein-coupled receptors (GPCRs) after ligand binding and phosphorylation, thereby promoting receptor desensitization, internalization, and signal transduction (1). In the brain, GPCR signaling is involved in slow synaptic neurotransmission, a process particularly important for long-term neuroadaptation and plasticity, both of which underlie fundamental cognitive and psychological processes, such as learning, memory, affective state, and reward (2). Among the four members of the arrestin family, beta-arrestins 1 and 2 are expressed throughout the brain. Although they have partly overlapping functions, it has also been shown that preferential recruitment of the respective arrestin is highly dependent on receptor and ligand (3, 4). In contrast to beta-arrrestin 1, *Arrb2* interacts with receptors that mediate rewarding properties of common drugs of abuse, such as μ-opioid and dopamine D2 receptors (5–8). For example, mice lacking *Arrb2*, but not mice without beta-arrestin 1, show increased sensitivity to morphine, a prototypical opiate that acts mainly through μ-opioid receptors (8). The effects of Arrb2 on μ-opioid and dopaminergic signaling are classically attributed to its role in ligand-induced receptor desensitization, but more recently, the expression of psychostimulant actions has been shown to be mediated through an Arrb2-dependent formation of a kinase/phosphate scaffolding complex at dopamine D2 receptors (6). Although these and other results suggest a role for beta-arrestin 2 in substance dependence, there are presently no data on how voluntary drug intake is modulated by variation in *Arrb2* expression and function.

Ethanol is consumed to millimolar concentrations in humans and is reinforcing in experimental animals. Its use is a major cause of morbidity and mortality. Recent data indicate that alcohol use accounts for ~85,000 deaths/yr in the United States alone, making it the third externally modifiable cause of mortality (9). The most severe form of alcohol use disorder, alcohol dependence, or alcoholism, occurs in ~5% of the adult population and has a 50–60% heritability. This is thought to be contributed by multiple loci, and the contribution of individual candidate genes accounts for not more than 5–10%, in a pattern common for complex polygenetic disorders resulting from gene-by-environment interactions (10, 11). Identification of heritable susceptibility factors might offer improved

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and ultimately individualized pharmacological treatment for this disorder.

In contrast to most other drugs of abuse, ethanol does not have a defined site of pharmacological action, but it is currently thought to act on specific proteins rather than *via* nonspecific membrane perturbations (12). Ethanol's reinforcing properties result, in part, from interactions with opioid and GABA neurotransmission within the mesolimbic dopaminergic pathway (13). Although *Arrb2* is involved in signaling through both the opioid and the dopaminergic components of this cascade, its role in ethanol consumption and reward has not been addressed. A link between ethanol reward and *Arrb2* was suggested by our prior microarray experiments involving the brains of experimental animals. These data pointed to genetic variation at this gene locus between two rat lines, the AA and ANA rats, selectively bred for high ethanol preference or ethanol avoidance, respectively (14, 15).

To investigate a possible role of *Arrb2* in ethanol reward, we resequenced the *Arrb2* locus in AA and ANA rats and found complete segregation between the lines at this locus. We discovered a novel haplotype variant in AA rats that spans the entire *Arrb2* locus. This genetic variant was associated with higher *Arrb2* transcript levels in several brain regions known to control alcohol reward and preference. Western blot analysis demonstrated that the differential gene expression translates into increased Arrb2 protein levels in the AA line. To perform a functional validation of the genetic findings, we studied the consumption and psychostimulant action of ethanol in mutant mice lacking *Arrb2*.

MATERIALS AND METHODS

Animals

All animal care and handling were in accordance with U.S. National Institutes of Health Guidelines. We obtained drug-naive male AA and ANA rats from the National Public Health Institute, Helsinki, Finland (15). Rats were of the 95th generation, aged 3 mo and housed 4/cage with food and water *ad libitum* on a reversed 12 h light/dark cycle (lights on at 10 PM).

The generation of *Arrb2* knockout mice was described previously (8). Mutants were generated on a C57BL/6 background. Genotyping of mice was performed using PCR from tail samples. Mutants and wild-type littermates were housed together in standard Plexiglas cages, with food and water *ad libitum* and on a 12 h light/dark cycle (lights on at 7 AM). Only males 2–3 mo of age were used in these experiments.

Sequence analysis

For sequence analysis, rats (n=15/line) were sacrificed, and blood was collected into EDTA tubes and stored at −20°C until DNA isolation. Genomic DNA was isolated from 3 ml of whole blood by using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). Approximately 1.6 kb of the promoter region and all exons (except for exon 11, which we were unable to amplify) were searched for sequence variation in a subset of 8 AA and 8 ANA rats. Sequencing was done as described; for primer sequences, see Supplemental Table 1 (16). Where evidence for sequence variation was found, the whole sample set was analyzed.

In situ hybridization

For *in situ* hybridization, animals were sacrificed by decapitation; brains were quickly removed, snap-frozen in −40°C isopentane, and stored at −70°C until use. *Arrb2* expression in AA and ANA rats was assessed in 8 animals/line. Brain sections were taken at bregma levels *1*) +2.5 to + 1.7 mm, *2*) −0.3 to −0.4 mm, *3*) −1.7 to −2.0 mm, and *4*) −2.3 to −3.3 mm, according to the atlas of Paxinos and Watson (17). The *in situ* hybridization protocol has been described previously (18). Sense and antisense riboprobes for the *Arrb2* and *c-fos* transcripts correspond to nucleotides 1238–1679 (NM_012911.1) and 306–864 (NM_022197.2), respectively. Fuji BAS-5000 PhosphorImager plates (Fujifilm, Tokyo, Japan) were exposed to the hybridized sections, and PhosphorImager-generated digital images were analyzed using AIS Image Analysis Software (Imaging Research Inc., St. Catharines, ON, Canada). Regions of interest were defined using anatomical landmarks as described in the atlas. On the basis of the known radioactivity in the ^{14}C standards, image values were converted to nCi/g. Expression values were compared regionwise by 1-way ANOVA followed by Holm's sequentially rejective multiple-test procedure.

Western blot analysis

Tissue samples from hippocampus of AA and ANA rats (n=6/strain) were prepared as described previously (19). Samples containing 35 μg total protein were separated on a 10% tris-glycine gel and blotted onto nitrocellulose membranes using the Xcell II system (Invitrogen, Carlsbad, CA, USA). Arrb2 protein was detected using an anti-Arrb2 antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA) and a mouse-specific secondary antibody (Bio-Rad, Hercules, CA, USA) and alpha-tubulin using a primary antitubulin antibody (Cell Signaling Technology Inc., Beverly, MA, USA) and a secondary anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Detection and quantitation were performed using the Fuji LAS-3000 system and Multigauge software (Fujifilm). The *Arrb2* and alpha-tubulin signal was normalized against total protein content as measured by Memcode staining (Pierce, Rockford, IL, USA) (19). Protein levels were compared by 1-way ANOVA.

Bioinformatic analysis

Quantitative trait locus analysis to define genomic regions associated with the expression of distinct genes (eQTL) was done using the WebQTL program at www.genenetwork.org (20). Linkage with *Arrb2* expression is expressed in logarithm of odds (LOD) scores and was plotted against chromosomal position. Seven independent data sets from recombinant inbred mouse lines between the C57BL/6J and DBA/2J parental lines (BXD RI strains were used): *1*) UCHSC BXD whole brain, from the University of Colorado at Denver; *2*) OHSU/VA B6D2F2 brain and *3*) OHSU/VA B6D2F2 striatum, from The Oregon Health Sciences University; *4*) HQF BXD striatum, from the University of Tennessee; *5*) the Hippocampus Consortium; *6*) VCU BXD prefrontal cortex, from Virginia Commonwealth University; and *7*) GE-NIAAA cerebellum.

Voluntary ethanol consumption

Increasing concentrations of ethanol in a 0.2% saccharin solution were made available in a continuous two-bottle free choice between ethanol-saccharine and saccharine-alone solutions. Bottles and mice were weighed on Mondays, Wednesdays, and Fridays between 9 and 10 AM to follow consumption. Bottle positions were switched daily to avoid development of side preference. Ethanol concentration was increased gradually from days 2 to 4 (2% ethanol+0.2% saccharin), to days 4 to 6 (4% ethanol+0.2% saccharin), and to days 7 to 28 (6% ethanol+0.2% saccharin). To control for putative differences in taste preference, the same mice were allowed *ad libitum* access to either sucrose (5%) or quinine (0.02 mM) for 14 days, and taste preference ratios were measured.

Ethanol-induced locomotion

Locomotion was assessed after a single injection of ethanol (0.75 g/kg). Infrared locomotor cages were used (27.9×27.9 cm test environment with three 16-beam I/R arrays; Med-Associates, St. Albans, VT, USA), and activity was recorded for 30 min in 10-min intervals. Results were analyzed using 2-way ANOVA for treatment and strain and Tukey's HSD *post hoc* test.

Ethanol metabolism and clearance

Blood levels of ethanol were measured after a single ethanol injection (3.5 g/kg, i.p.). Blood samples were obtained from the tail at 15, 60, 120, and 240 min after injection. The samples were subsequently analyzed using Analox analyzer (Analox Instruments, Luneburg, MA, USA). Data were analyzed using repeated-measures ANOVA.

Rotarod

Four-month-old mice, with and without *Arrb2*, were placed on a rotarod (Ugo Basile, Varese, Italy) turning at a fixed rate of 10 rpm. The mice learned to remain on the rod during three 60-s training periods. Saline or increasing doses of alcohol (0.5 mg/kg dose, range from 0.5 to 3 mg) were injected i.p. After 5 min, each mouse was tested 3 times in succession for its ability to remain on the rod. The cutoff time was 60 s. The ED_{50} was calculated using nonlinear regression implemented in GraphPad Prism v4 (GraphPad Software Inc., San Diego, CA, USA) and compared by 1-way ANOVA.

RESULTS

Sequencing of *Arrb2* in AA and ANA rats revealed complete segregation of two genetic variants between the lines (**Fig. 1*A***). The AA-specific, high ethanol preference-associated haplotype, herein referred to as var1, consisted of 7 polymorphisms (6 SNPs and 1 insertion), which were in complete linkage disequilibrium (LD). The sequence of *Arrb2* in ANA animals did not differ from the published *Rattus norvegicus* sequence. $Arrb2^{var1}$ is the only genetic variant discovered to date that segregates entirely between the AA and ANA lines.

In situ hybridization demonstrated that the presence of $Arrb2^{var1}$ in the ethanol-preferring AA line was associated with significantly higher *Arrb2* mRNA levels in the nucleus accumbens, dorsal striatum, and hippocampus (Fig. 1*B*). A corresponding difference in hippocampal Arrb2 protein levels was detected using Western blot, while the housekeeping gene alpha-tubulin was unaltered between the two lines. (Fig. 1*C*).

Next, we asked whether genetic variation at the *Arrb2* locus could affect a *cis*-regulatory mechanism for expression and regional control of the gene. Bioinformatic analysis in seven independent microarray expression data sets, including samples from whole brain, prefrontal cortex, hippocampus, striatum, or cerebellum of BXD RI strains revealed a single, distinct, and highly significant eQTL for *Arrb2*, which peaks above its own locus on mouse chromosome 11. The highest linkage at the *Arrb2* locus was found in samples from hippocampus and striatum (LOD scores 9.8 and 8.2, respectively. LOD scores above 3.3 are considered significant, **Fig. 2**). Prefrontal cortex and two whole brain data sets showed less significant or suggestive eQTLs, with LOD scores of 5.1, 4.3, and 2.5, respectively. No evidence for an eQTL at the *Arrb2* locus was found in the samples from cerebellum. Similar eQTLs are not found at *Arrb2*'s closest neighbor genes, demonstrating that the effect is specific for *Arrb2*. Furthermore, no eQTL exists for beta-arrestin 1 in any of the data sets.

To establish a functional relationship between *Arrb2* and ethanol consumption, we used mutant mice lacking *Arrb2* (8). Both homozygote and heterozygote *Arrb2* mutants ($Arrb2^{-/-}$ and $Arrb2^{+/-}$) consumed significantly less and had lower preference for ethanol than wild-type littermate controls when ethanol was available in a two-bottle free-choice paradigm (6% vol/vol ethanol/0.2% saccharine or 0.2% saccharine only in tap water; **Fig. 3*A–C***). There was a reciprocal effect on intake from the nonethanol bottle, and total liquid intake did not differ between the genotypes, suggesting that nonspecific differences in regulation of liquid intake and thirst did not account for the reduced ethanol consumption in the mutant mice. There were no significant weight differences at the end of the experiment: 27.6 ± 0.68, 27.1 ± 0.57, and 27.4 ± 0.75 for $Arrb2^{-/-}$, $Arrb2^{+/-}$, and $Arrb2^{+/+}$ animals, respectively.

Pharmacological specificity for ethanol was further supported by the taste preference experiments. Alcohol's taste is thought to be a composite of sweet and bitter. An absence of differences in taste preferences for sucrose and quinine between the genotypes demonstrated that the differences in ethanol intake between genotypes are not likely to be driven by altered taste preference (Fig. 3*D, E*).

The ethanol metabolism and elimination experiment demonstrated that drinking differences between genotypes are not driven by pharmacokinetic differences. $Arrb2^{-/-}$ mice did show somewhat lower blood ethanol levels after systemic administration of 3.5 g/kg ethanol compared to wild-type mice, whereas $Arrb2^{+/-}$

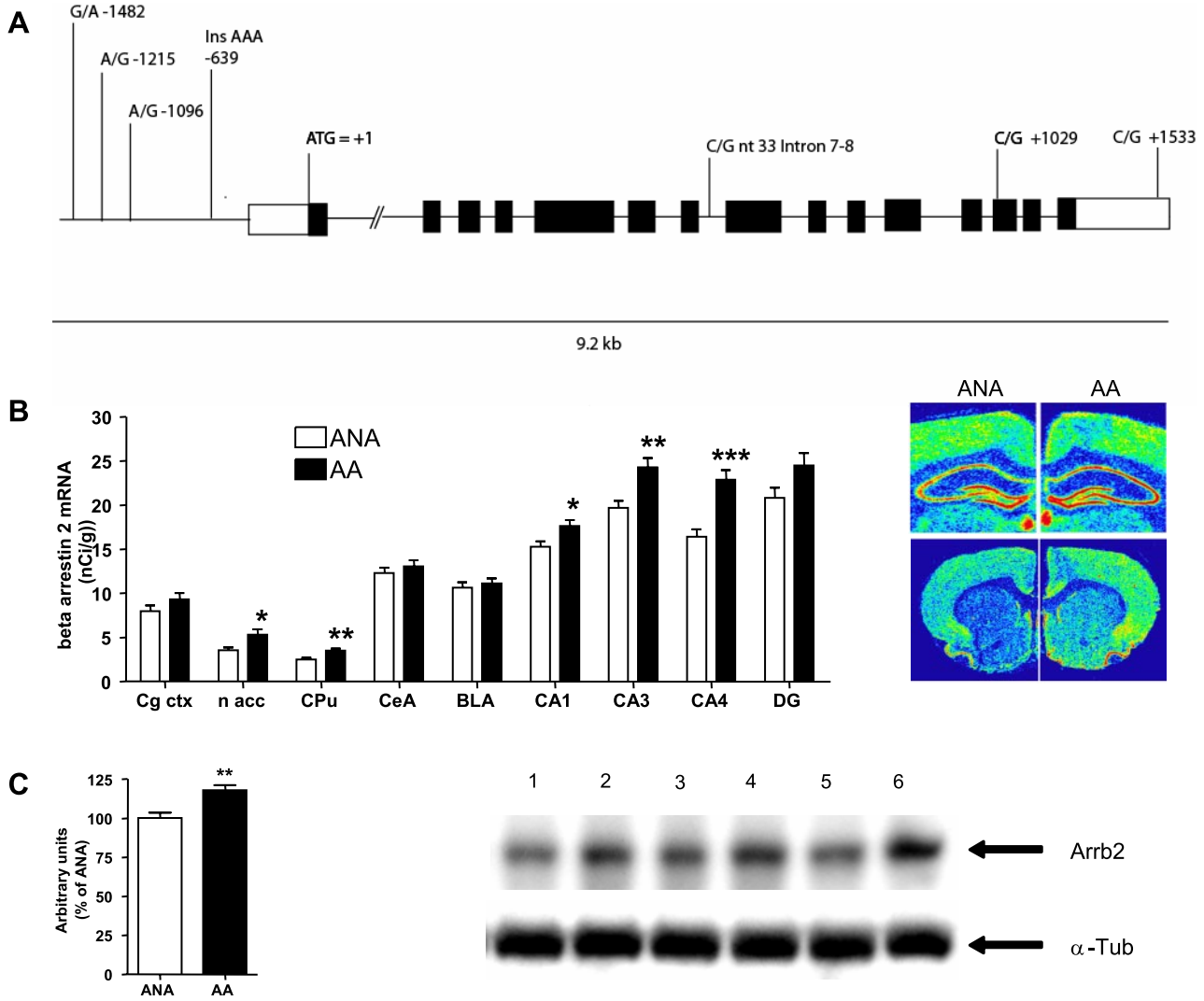

Figure 1. Genetic variation at the Arrb2 locus is associated with increased gene expression. *A*) AA rats are homozygous for a novel haplotype block (analysis by Haploview), comprising 1 insertion and 6 single nucleotide polymorphisms (SNPs). The ANA line carries the wild-type sequence, according to the ENSEMBL database (ENSRNOG00000019308). No other genetic variation was detected. The nucleotide position is calculated from the A in the start codon of the mRNA sequence (NM_012911.1), which is designated as +1. Positions in the promoter are calculated in the 5′ direction starting from the nucleotide preceding the start codon, which is designated as −1. Exons are shown as boxes; the coding region is black, and untranslated regions are white. SNP positions are indicated by lines, with the wild-type allele given first. Introns are not shown at full length. Loci of 15 animals/line were sequenced. *B*) *In situ* hybridization shows elevated *Arrb2* mRNA levels in AA as compared to ANA rats. Expression values were compared regionwise by 1-way ANOVA followed by Holm's sequentially rejective multiple test procedure. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 8$ animals/line. Right panel: representative autoradiogram of coronal brain sections showing increased *Arrb2* expression in hippocampus (top) and dorsal and ventral striatum (bottom). Cg ctx, cingulate cortex; CPu, caudate putamen; n acc, nucleus accumbens; CeA, central amygdaloid nucleus; BLA, basolateral amygdaloid nucleus, CA1 to CA4, Cornus Ammon areas; DG, dentate gyrus. *C*) Western blot analysis revealed elevated *Arrb2* protein levels in the hippocampus of AA rats compared to ANA rats. Protein levels of the housekeeping gene alpha-tubulin were unaltered between the two strains. Densitometric measures of both proteins were normalized to the mean of the ANA group and analyzed using 1-way ANOVA $**P < 0.01$; $n = 6$ animals/line. Representative Western blots are shown to the right. Lanes 1, 3, and 5 are samples from ANA rats, lanes 2, 4, and 6 from AA rats.

mice did not **(Fig. 4*A*)**. Since reduced voluntary ethanol intake was found in both *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice, differences in ethanol metabolism and elimination cannot account for the drinking findings. Similarly, sensitivity to ethanol's motor-impairing effects was somewhat reduced in heterozygous mice only, and is therefore unlikely to account for the reduced ethanol drinking found both in *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice (Fig. 4*B*). Similarly, the previously reported lower spontaneous locomotor activity of *Arrb2* mutant mice (5, 6) is unlikely to account for the observed differences in ethanol drinking and sensitivity.

Psychomotor stimulation, resulting in increased locomotor activity, is a proxy measure of mesolimbic dopaminergic system activation. Most drugs of abuse, including ethanol, can induce locomotor activity in

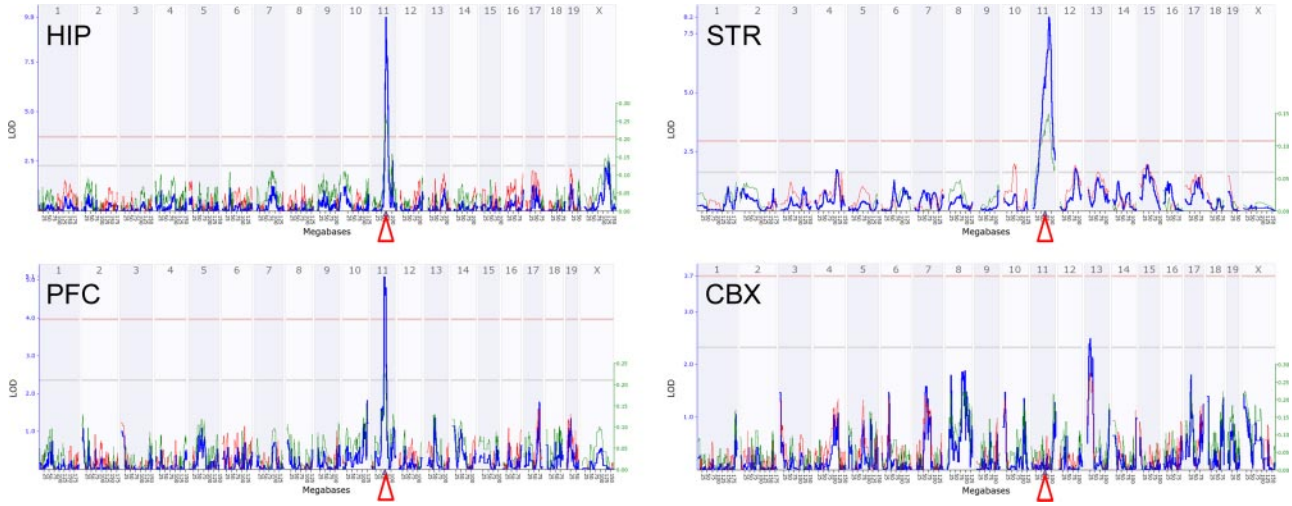

Figure 2. Representative graphs from eQTL analysis of brain tissue of recombinant inbred mouse lines of the BXD panel. The blue line shows the likelihood of linkage with *Arrb2* expression in LOD scores (*y* axis) at a given chromosomal position (*x* axis). A single linkage peak with *Arrb2* expression was found in several brain regions. Threshold for significant and suggestive LOD scores are shown as horizontal red and gray lines, respectively. Triangles point to the position of the *Arrb2* locus on mouse chromosome 11. Results from hippocampus (HIP), striatum (STR), prefrontal cortex (PFC), and cerebellum (CBX) are shown. Analysis was done using the WebQTL program at www.genenetwork.org (20).

experimental animals. In line with the findings of reduced voluntary ethanol drinking, *Arrb2* mutants were insensitive to the stimulant effects of ethanol (0.75 g/kg; **Fig. 5*A***). Consistent with the absence of ethanol-induced locomotion in *Arrb2*$^{-/-}$ mice, ethanol-induced *c-fos* expression in the shell of nucleus accumbens was also absent in homozygous mutants (Fig. 5*B*). No differences in *c-fos* expression were found in the core of the nucleus accumbens. Other regions were not studied.

DISCUSSION

We found elevated *Arrb2* expression in the nucleus accumbens and hippocampus of high-ethanol-preferring AA rats compared to ANA rats, associated with a novel haplotype variant of the *Arrb2* gene that showed complete segregation between the preferring and non-preferring line. Mice lacking one or both copies of *Arrb2* showed reduced voluntary ethanol consumption compared to their wild-type littermates, and this was

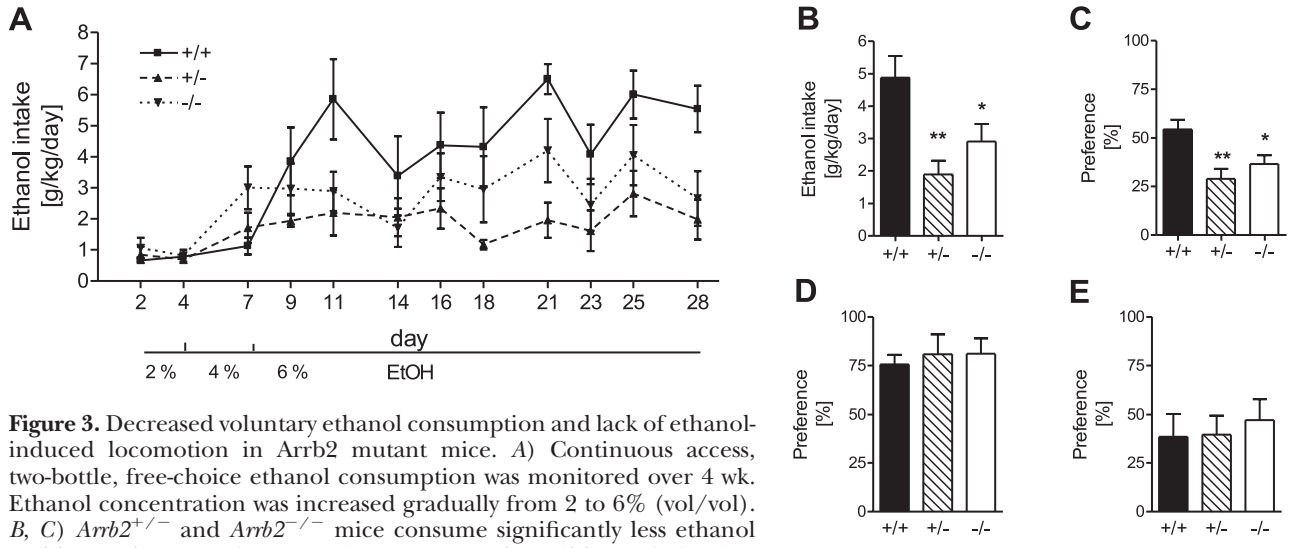

Figure 3. Decreased voluntary ethanol consumption and lack of ethanol-induced locomotion in Arrb2 mutant mice. *A*) Continuous access, two-bottle, free-choice ethanol consumption was monitored over 4 wk. Ethanol concentration was increased gradually from 2 to 6% (vol/vol). *B, C*) *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice consume significantly less ethanol and have a lower preference ratio as compared to wild-type (wt) mice. Mean alcohol intake for the 6% ethanol solution was analyzed by 1-way ANOVA: $F_{(2, 22)} = 7.1$, $P < 0.01$; Fisher's *post hoc* test *vs.* *Arrb2*$^{+/+}$: $^{*}P < 0.05$; $^{**}P < 0.01$. Ethanol preference: $F_{(2, 22)} = 7.0$, $P < 0.01$; Fisher's *post hoc* test *vs.* *Arrb2*$^{+/+}$: $^{*}P < 0.05$; $^{**}P < 0.01$. There was no statistical difference between *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice. A reciprocal increase in consumption from the nonethanol bottle, but not in total fluid intake, was observed. *D, E*) Taste preference for sucrose (5%) and quinine (0.02 mM), respectively. Mice were allowed access to sucrose and quinine in a two-bottle, free-choice, continuous access paradigm *vs.* tap water for 7 days, and taste preference ratios were acquired. Results were analyzed using 1-way ANOVA. There were no significant differences between wild-type and *Arrb2* mutant mice.

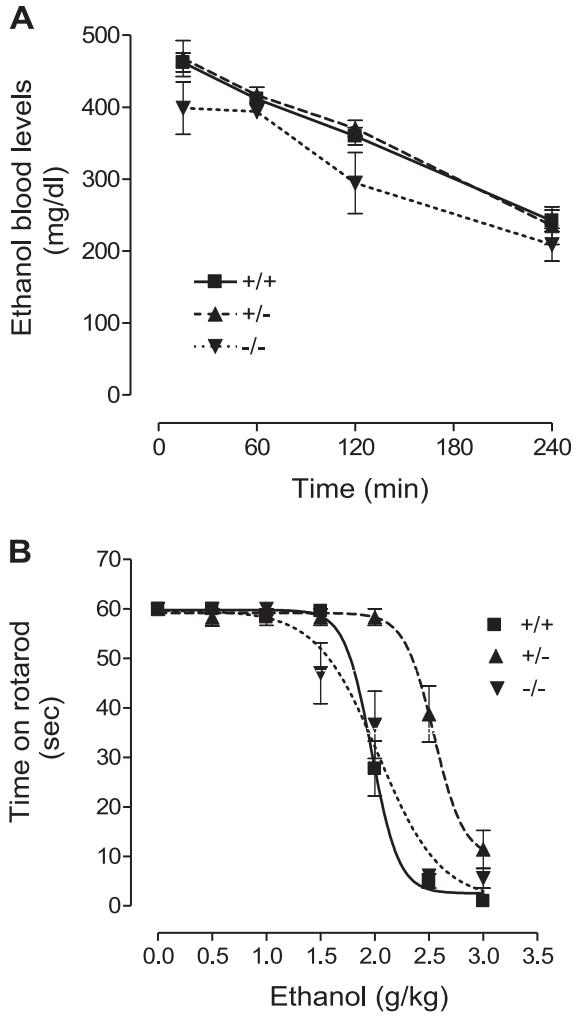

Figure 4. Ethanol clearance and sedation in *Arrb2* mutant mice. *A*) Blood ethanol levels were measured at 15, 60, 120, and 240 min following a single ethanol injection (3.5 g/kg, i.p.). *Arrb2*$^{-/-}$ mice display significantly lower blood ethanol levels as compared to both *Arrb2*$^{+/-}$ and *Arrb2*$^{+/+}$ mice. However, there were no differences in clearance rates between genotypes. Results were analyzed using repeated measurement ANOVA for genotype and time. Genotype: $F_{(2, 10)} = 15.2$, $P < 0.001$; time: $F_{(3, 30)} = 43.3$, $P \ll 0.001$; interaction: $F_{(6, 30)} = 0.3$, not significant. Tukey's HSD *post hoc* test: $P < 0.01$, *Arrb2*$^{-/-}$ *vs.* both other genotypes. *B*) Motor performance on the rotarod was used to assess the sedating effects of ethanol. Successive injections of ethanol (0.5g/kg every 5 min, i.p.) were given for 30 min; the animal was placed on the rotarod 5 min after each injection, and the time that elapsed until the animal fell off the rod was recorded. ED_{50} was calculated for each animal and compared for statistical significance using 1-way ANOVA. *Arrb2*$^{+/-}$ mice exhibit a significantly slower onset of sedation compared to *Arrb2*$^{-/-}$ and *Arrb2*$^{+/+}$ mice. Mean ED_{50} for *Arrb2*$^{-/-}$, *Arrb2*$^{+/-}$, and *Arrb2*$^{+/+}$ = 1.985 ± 0.08, 2.654 ± 0.06, and 1.935 ± 0.06, respectively.

unlikely to be caused by differences in taste preference or ethanol metabolism. Together, these data provide evidence for a role of *Arrb2* in mediating ethanol reinforcement.

A number of rodent lines have been successfully bred for different ethanol preference and are used in work aimed at identifying targets for novel pharmacological treatments for alcohol dependence (21, 22). The alcohol-preferring AA and the alcohol-avoiding ANA rat lines have been bred bidirectionally for high and low alcohol consumption, respectively, for more than 90 generations (15). AA rats display some interesting behavioral traits characteristic of early onset alcoholism

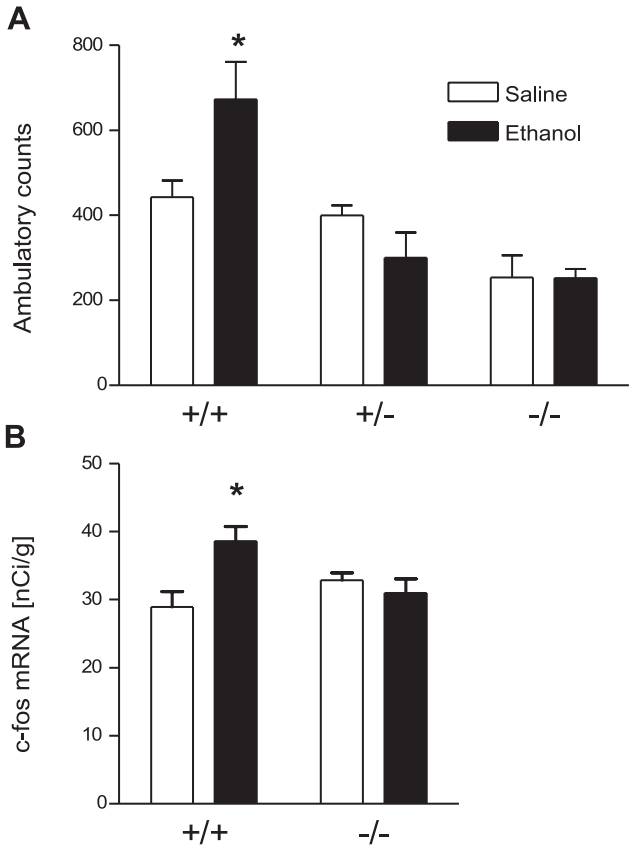

Figure 5. Decreased ethanol-induced locomotion and *c-fos* response in *Arrb2*$^{-/-}$ mice. *A*) Locomotor activity was measured after a single administration of ethanol (0.75 g/kg, i.p.). Wild-type animals displayed a significant increase in locomotion subsequent to ethanol injection. Ethanol lacks stimulant properties in *Arrb2*$^{-/-}$ mice. Two-way ANOVA for genotype and ethanol treatment: genotype: $F_{(2, 37)} = 17.0$, $P < 0.0001$; ethanol: $F_{(1, 37)} = 0.9$, $P = 0.342$; interaction: $F_{(2, 37)} = 4.9$, $P = 0.013$. The significant genotype effect agrees with earlier observations (5) that *Arrb2* knockout mice display lower spontaneous locomotor activity compared to wild-type mice. However, the lack of ethanol-evoked hyperlocomotion in *Arrb2* mutant mice is not explained by a general impairment in spontaneous locomotor activity as demonstrated by Tukey's HSD *post hoc* analysis: except for ethanol-treated *Arrb2*$^{+/+}$ animals, no significant differences between groups were found. *B*) Ethanol-induced *c-fos* expression is diminished in *Arrb2* mutant mice. *In situ* hybridization shows elevated *c-fos* mRNA levels in the nucleus accumbens shell (bregma level: 1.7–1.5) of wild-type animals 45 min after a single injection of ethanol (0.75 g/kg, i.p.) as compared to saline-injected controls. Two-way ANOVA for genotype and ethanol: genotype $F_{(1, 13)} = 0.8$, not significant; ethanol $F_{(1, 13)} = 3.6$, not significant; interaction $F_{(1, 13)} = 7.9$, $P = 0.015$. Tukey's HSD *post hoc* test: $P < 0.05$, ethanol *vs.* saline in *Arrb2*$^{+/+}$. Data are expressed as means ± SE. *$P < 0.05$, wild-type ethanol *vs.* saline groups.

in humans, which has a distinct heritability and pharmacogenetic profile (23, 24). The AA and ANA lines have diverged with respect to a variety of behavioral and neurochemical measures, but the factors causing their differential ethanol preference remain largely unknown (15).

All candidate genes previously screened for allelic variation between AA and ANA rats have shown incomplete segregation between the two lines, making it difficult to establish a link between genetic findings and a behavioral or expression phenotype (16). The complete segregation of AA and ANA animals at the *Arrb2* locus into two haplotype variants is a unique finding for noninbred lines and suggests biological significance. The length of the novel haplotype block spans the entire *Arrb2* locus, but it is unclear whether a high degree of LD extends beyond this locus. The average length of a haplotype block in rats is currently not known. Furthermore, it is difficult to obtain block size estimates for selected populations because block size is strongly dependent on the genetic diversity of the founder population and will be severely skewed by any random fixation effects that occur during selection. Although this leaves open the possibility for an involvement of neighboring genes in the biological effects of the variant allele, we find several lines of evidence that favor an important role for *Arrb2*.

First, the *in situ* hybridization experiment reveals differences in *Arrb2* expression between AA and ANA rats in the hippocampus and striatum, two brain regions known to be important in substance dependence (25). Furthermore, albeit the differences in *Arrb2* expression are modest and restricted to distinct brain regions, their coexistence with widespread genetic variation at the gene locus points to *cis*-acting mutations, probably altering regulatory elements involved in regional control of gene expression.

Second, a *cis*-regulatory mechanism for expression and regional control of *Arrb2* is supported by bioinformatic analysis. Combination of expression profiling from recombinant inbred mouse lines of the BXD RI panel and genome-wide allelic marker association allow identification of genomic regions that control the expression of individual genes (26, 27). This approach was recently employed to identify novel candidate genes involved in ethanol preference and tolerance (28). Here, we found a single, distinct, and highly significant eQTL for *Arrb2* at its own locus in hippocampal, striatal, and prefrontocortical tissue but not in cerebellum. These findings strongly suggest that variation at the *Arrb2* locus has region-specific impact, and it is important for *Arrb2* expression in the hippocampus and striatum, the regions that show the largest *Arrb2* expression differences between AA and ANA rats. Furthermore, no eQTL exists for beta-arrestin 1 in any of the data sets, suggesting that *Arrb1* and *Arrb2* are regulated through independent mechanisms. It remains unknown for now how the polymorphic alleles of *Arrb2* contribute to the regulation of its gene expression.

Third, an involvement of a candidate gene in alcohol preference can be supported by a consistent pattern of differential gene expression across different rodent lines with differing ethanol preference. Notably, significant and consistent up-regulation of brain *Arrb2* expression was found by microarray analysis in selected, noninbred mouse lines with high ethanol preference compared to their controls (29). However, differential *Arrb2* expression was not found in the brains of another widely studied pair of rat lines, the Indiana ethanol-preferring P and nonpreferring NP rats, nor does the *Arrb2* gene reside in any of the QTLs for ethanol preference that have been identified in rodents so far (reviewed in ref. 28).

Finally, the most straightforward functional validation for a differentially expressed candidate gene can be obtained by interventions that disrupt the function of its product. When available, we have used pharmacological tools to investigate the role of candidate genes identified on the basis of their differential expression between high- and low-preferring animals, such as endocannabinoid genes in AA rats (30). However, in the absence of suitable pharmacological tools or transgenic approaches for probing the function of *Arrb2* directly in AA rats, we used a genetic strategy using *Arrb2* knockout mice. Ethanol preference is affected in numerous mutant lines, suggesting that the control of this behavior is complex and may also be affected by behaviorally nonspecific effects of the mutations (31). Consequently, we tested for and excluded a number of possible confounding factors that might contribute to the reduced ethanol consumption in mutants lacking *Arrb2*. Neither taste preference nor ethanol metabolism or sedation seemed to account for the reduced ethanol consumption in *Arrb2* knockout mice. Importantly, both mice homozygous and heterozygous for the *Arrb2* null mutation exhibited markedly reduced ethanol drinking. This finding underscores that ethanol preference and reward are sensitive to changes in *Arrb2* expression and function.

The mechanism of action of *Arrb2* in modulation of ethanol reward remains unknown. Dopamine-opioid system interactions contribute to ethanol reward (13, 32). Endogenous opioids released by ethanol can act at μ-opioid receptors located on midbrain GABAergic interneurons, leading to disinhibition of dopaminergic neurons in the ventral tegmental area, and dopamine release into their terminal regions (33–36). In addition, similar to morphine, ethanol might act directly on the target neurons of the mesocorticolimbic pathway (37), since inactivation of the mesocorticolimbic dopamine neurons does little to modulate ethanol self-administration (38, 39), while pharmacological blockade or gene knockout of μ-opioid receptors substantially reduces ethanol consumption and ethanol-induced locomotion (40–42).

Arrb2 expression and function appear to be negatively correlated with analgesic effects of opioids, while its role in opioid-mediated reward is less consistent. Deletion of *Arrb2* in mice potentiates and prolongs

morphine analgesia (7, 8), an effect partially rescued by local overexpression of *Arrb2* in brain regions involved in pain control (43). However, although both conditioned place preference for opiates, a measure of reward, and exracellular dopamine release in response to morphine are increased in the *Arrb2* mutants, morphine-induced locomotor activity is decreased in these animals (5). No drug self-administration data are previously available from *Arrb2* mutants, and the conditioned place preference data are therefore the only ones that directly address how opioid reward is modulated by *Arrb2*. These results suggest that, similar to opioid analgesia, opioid reward is inversely related with *Arrb2* expression and function. If that indeed is the case, the positive correlation between *Arrb2* expression and voluntary ethanol intake observed in AA rats and *Arrb2* mutants in the present study cannot be attributed to opioid mechanisms.

It has recently been shown that beta-arrestins can mediate receptor signaling independent of G proteins and the cyclic AMP cascade *via* a scaffolding complex with certain kinases and/or phosphatases (44). This mechanism appears to be crucial for psychomotor stimulation following activation of dopamine D2 receptors. Psychostimulant-induced locomotor activity depends on the formation of a signaling complex at the D2 receptor, which includes *Arrb2* and the kinases PP2A and Akt (6). It has been suggested that this mechanism underlies the profound reduction in locomotor response to amphetamine and morphine in *Arrb2* mutants (5, 6, 45). Here, we show that ethanol-stimulated activity is also reduced, and disruption of noncanonical D2 signaling could account for this observation. Activation of the ventral striatum plays a crucial role in psychostimulant-induced locomotor behavior (46). The actions of psychostimulants, ethanol, and opiates, are all associated with induction of the immediate early gene *c-fos* in these regions (47–51). Although this gene is induced by a variety of stimuli, the *c-fos* response has been causally linked to locomotor response to psychostimulants (52–54). Consistent with these findings, *c-fos* induction after acute systemic challenge with a low dose of ethanol was absent in *Arrb2* knockout mice. A possible implication of this finding is that ventral striatal *c-fos* induction by ethanol is downstream of the recently postulated noncanonical *Arrb2* signaling cascade.

Also, since the most robust differences in *Arrb2* expression between AA and ANA animals are found in the hippocampus, the possibility exists that this region is involved in ethanol preference in these lines and in *Arrb2* mutant mice. In fact, ANA rats have impaired learning and memory compared to AA animals. However, the differences in alcohol drinking reported here were assessed in a simple task that does not require learning. Furthermore, the large body of literature on these lines does not give any indication that memory impairment is a key factor explaining the divergence in ethanol and drug-related behaviors between the lines (15).

In summary, we report that *Arrb2* expression is positively correlated with ethanol preference, suggesting a role for this protein in mediation of ethanol reward within the mesocorticolimbic system. FJ

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